

# Vitellogenin Induction and Reduced Serum Testosterone Concentrations in Feral Male Carp (*Cyprinus carpio*) Captured Near a Major Metropolitan Sewage Treatment Plant

Leroy C. Folmar,<sup>1</sup> Nancy D. Denslow,<sup>2</sup> Vijayasri Rao,<sup>2</sup> Marjorie Chow,<sup>2</sup> D. Andrew Crain,<sup>3</sup> Jack Enblom,<sup>4</sup> Joseph Marcino,<sup>4</sup> and Louis J. Guillette Jr.<sup>3,5</sup>

<sup>1</sup>U.S. Environmental Protection Agency, Gulf Breeze, FL 32561 USA; <sup>2</sup>Department of Biochemistry and Molecular Biology; and <sup>3</sup>Department of Zoology, University of Florida, Gainesville, FL 32610 USA; <sup>4</sup>Minnesota Department of Natural Resources, MN 55155 USA; <sup>5</sup>Center for Bioenvironmental Research, Tulane University, New Orleans, LA 70112 USA.

Endocrine disrupting chemicals can potentially alter the reproductive physiology of fishes. To test this hypothesis, serum was collected from common carp (*Cyprinus carpio*) at five riverine locations in Minnesota. Male fish collected from an effluent channel below the St. Paul metropolitan sewage treatment plant had significantly elevated serum egg protein (vitellogenin) concentrations and significantly decreased serum testosterone concentrations compared to male carp collected from the St. Croix River, classified as a National Wild and Scenic River. Carp collected from the Minnesota River, which receives significant agricultural runoff, also exhibited depressed serum testosterone concentrations, but no serum vitellogenin was apparent. These data suggest that North American rivers are receiving estrogenic chemicals that are biologically active, as has been reported in Great Britain. **Key words:** estradiol, fish, sewage effluent, testosterone, vitellogenin. *Environ Health Perspect* 104:1096–1101 (1996)

Concern for estrogenic effects of environmental xenobiotic chemicals on human and wildlife health has existed for over 25 years (1,2). However, within the last 4 years, this interest has become focused and intensified (3–5). Among wildlife species, research has been focused on animals associated with wetland or aquatic habitats receiving sewage or industrial effluent and agricultural runoff. As with most studies in aquatic toxicology, fish have received the greatest attention. In fish, estrogenic responses have been associated with exposure to pesticides (6), surfactants (7), pulp mill effluent/phytoestrogens (8–11), industrial waste (12), and sewage effluent (13,14). In these studies, one measure of estrogenic activity is the production of vitellogenin (VTG), an estrogen-inducible egg protein precursor. This protein, normally synthesized in the liver of female oviparous vertebrates, is estrogen dependent and increases markedly in the serum during oocyte development (15,16). In general, the VTG gene is present but not expressed in males [there are exceptions (17–21)]. However, administration of exogenous estrogen, estrogen mimics, or P450 aromatase inducers can elicit VTG synthesis in males (22–24). The primary purpose of this investigation was to determine whether male carp (*Cyprinus carpio*) collected in the Mississippi River below a major metropolitan sewage treatment plant (STP) exhibited elevated serum VTG concentrations and altered serum sex steroid concentrations when compared with a relatively pristine reference site, the St. Croix River. Either abnormality in male fish could

indicate the presence of estrogenic chemicals in their riverine environment. To determine the extent of any observed effect, we collected carp from two additional Mississippi River locations below the STP and from a Mississippi River tributary receiving agricultural runoff, the Minnesota River.

## Methods and Materials

### Fish Collection

Male carp were collected from five locations around the St. Paul, Minnesota, area (Fig. 1): 1) a natural side channel of the Mississippi River receiving effluent from the St. Paul Metropolitan Sewage Treatment Works (STP, river mile 835,  $n = 10$ ); 2) Mississippi River Navigational Pool #2 (MRP-2, between river miles 815 and 832,  $n = 10$ ); 3) Mississippi River Pool #3 (MRP-3, between river miles 798 and 802,  $n = 10$ ); 4) The Minnesota River, a Mississippi River tributary receiving extensive agricultural runoff (MNR, river mile 69,  $n = 10$ ); and 5) The St. Croix River, our reference site classified as a National Wild and Scenic River (SCR, between river miles 63 and 125,  $n = 5$ ). Additionally, female carp were collected from the effluent channel ( $n = 7$ ) and from MRP-2 ( $n = 10$ ). Fish were collected using a pulsed DC electrofishing boat. Fish, which were all in post-spawning condition, were obtained from the effluent channel during the last week of August and from the other locations between 20 September and 16 October 1995. River flow rates at the time of collec-

tion were > 200% of normal for this period. All fish were mature adults between ages III and V; mean length values are given in mm  $\pm$  1 standard error: STP, 472.2  $\pm$  5.9 male (M) and 466.6  $\pm$  9.3 female (F); MRP-2, 474.1  $\pm$  10.7 (M) and 523.8  $\pm$  14.5 (F); MRP-3, 500.2  $\pm$  11.4; MRR, 496.2  $\pm$  12.6; and SCR, 628.0  $\pm$  13.3. Blood was drawn by cardiac puncture into unheparinized vacutainers and allowed to clot. The samples were centrifuged and the serum was pipetted into 1.5-ml microfuge tubes pretreated with aprotinin and frozen at -70° until analyzed.

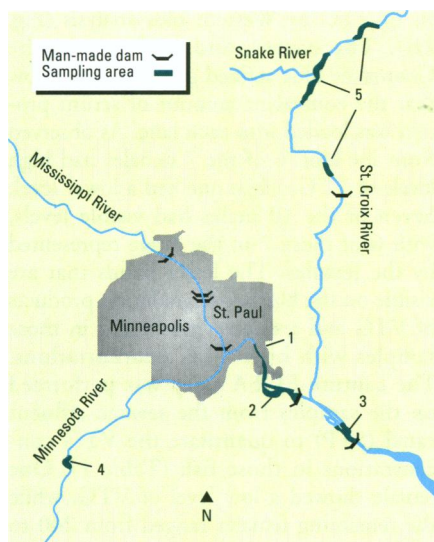
### Vitellogenin Analysis

The monoclonal antibody Mab HL 1147 (2D3-3A9) was raised against a mixture of fish vitellogenins injected into a Balb/C mouse in the Hybridoma Core Facility at the University of Florida. Briefly, a mouse was injected subcutaneously at three sites, first with purified striped bass VTG and then twice with a mixture of acrylamide gel pieces of purified brown bullhead and carp VTG in RIBI MPL and TDM adjuvant (RIBI Immuno Chemical, Inc., Hamilton, MT) at 2- to 3-week intervals. The mouse was then injected with a combination of 75  $\mu$ g of purified carp VTG and gel pieces of carp and brown bullhead VTG, followed by a final intraperitoneal (ip) boost with 75  $\mu$ g of purified carp VTG. Hybridomas were screened against carp, striped bass, and brown bullhead VTG by ELISA. We obtained hybridomas for carp and striped

Address correspondence to L. C. Folmar, U.S. Environmental Protection Agency, 1 Sabine Island Drive, Gulf Breeze, FL 32561 USA.

The authors wish to thank the following individuals for their assistance in sample collection and preparation: Ranjit Bhagyam, Michael Feist, Bonnie Mallory, Ken Mueller, and Dan Orr (Northern States Power Company). This work was funded in part by a U.S. Environmental Protection Agency (EPA) Cooperative Agreement (CR821437) to L.J.G. and N.D.D., grants to L.J.G. (PR471437) and N.D.D. (E507375) from the NIEHS, grants to L.J.G. from the EPA (R824760-01-0) and the Alton Jones Foundation, and an EPA graduate fellowship to DAC (U-914738-01-0). Mention of trade names does not constitute government endorsement.

Received 20 February 1996; accepted 20 June 1996.



**Figure 1.** Map depicting the five collection sites. 1) A 1250 ft channel below the St. Paul metropolitan sewage treatment plant; 2) Mississippi River Navigation Pool #2; 3) Mississippi River Navigation Pool #3; 4) Minnesota River; and 5) St. Croix River.

bass VTG but not for brown bullhead VTG, indicating that injecting purified VTG gel pieces was not as effective as injecting purified VTG in liquid form. Mab HL 1147 (2D3-3A9), one of the hybridomas from the fusion, reacts specifically and has high affinity with carp VTG in sera of vitellogenic females or 17 $\beta$ -estradiol ( $E_2$ )-induced animals, but not with proteins in serum from non-induced males, as determined by Western blot analysis. Tissue culture supernatant, rich in monoclonal antibody, was used for the Western blot analyses. Mab HL 1147 (2D3-2A9) was purified from tissue culture supernatants by chromatography on a Protein-G column using Pierce Immunopure Ag/Ab

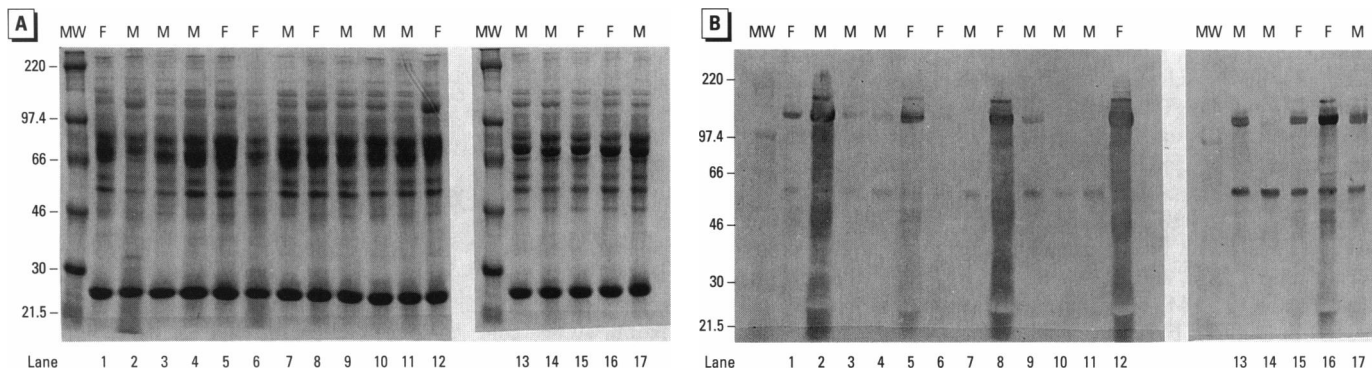
(Pierce Chemical Company, Rockford, IL) as the elution buffer. This purified antibody was used in the capture-ELISA assay.

A polyclonal antisera (UF114) was raised in rabbits by injection of a mixture of polyacrylamide gel-purified VTGs from several species including carp, largemouth bass, striped bass, bluegill sunfish, and rainbow trout. The rabbit was boosted with three injections of the above VTG cocktail minus the rainbow trout VTG. Before use, antiserum UF114 was mixed with one-sixth volume of serum from control males to remove nonspecific reactivity. Precipitated material was removed by high speed centrifugation. The clear supernatant contained antisera that was specific and of high affinity for carp VTG. This antisera was used in the quantitative capture ELISA described below. For gel electrophoresis and Western blot analyses, serum samples were diluted 25-fold with Laemmli sample buffer (25), and 15  $\mu$ l of each sample was applied in duplicate to preformed wells in 7.5% Tris-tricine acrylamide gels (26). Immediately after electrophoresis, one set of gels was stained with Coomassie Brilliant Blue (R250) to visualize the proteins, and another set of gels was electroblotted overnight at 20 V and 4°C onto Immobilon P membranes (Millipore Corporation, Bedford, MA) using 10 mM MES (morpholinoethane sulfonic acid), pH 6, 20% MeOH as the transfer buffer (27). For Western blot analyses, membranes were blocked with 5% non-fat dry milk (28) in TBST (10 mM Tris, pH 7; 150 mM NaCl; 0.5% Tween) for 2 hr at room temperature and then incubated directly with tissue culture supernatant containing Mab HL 1147 (2D3-3A9) for 16 hr at 4°C. The membrane was then washed in TBST and probed with goat anti-mouse alkaline phosphatase-conjugat-

ed secondary antibody (diluted 1:1000) for 2 hr at room temperature. Blots were developed by incubating them with bromochloroindolyl phosphate/nitro blue tetrazolium substrate. Carp VTG was detected as a set of two high-molecular weight proteins of 180 kDa (Fig. 2; thin band in blot) and 150 kDa (wide band). In addition, there were a number of degradation products of VTG detected by this sensitive assay in blots of samples with high levels of VTG. These bands make up less than 1% of the VTG protein and are not detected by Coomassie staining, thus illustrating the superior sensitivity of the Western blot assay.

### Capture-ELISA Assay

VTG was quantified using a capture-ELISA assay. Carp VTG, purified by chromatography on DEAE (diethylamino ethyl anion exchange medium; Perceptive Biosystems, Inc., Farmington, MA), was used as a standard. Protein concentration was determined by the Bradford assay (29). Purified Mab HL 1147 (2D3-3A9), diluted to a concentration of 10  $\mu$ g/ml in phosphate buffered saline (PBS), was coated onto the surface of 96-well microtiter plates (50  $\mu$ l/well) overnight at 4°C. The plates were washed with TBST and blocked with 360  $\mu$ l/well of 0.1% BSA in TBST for 2 hr at room temperature. Again, the plates were thoroughly washed with TBST. Fish plasma samples were diluted in the range of 1:500 to 1:5000 in 0.1% BSA in TBST. Fifty microliters of this dilution was added, in duplicate, to individual wells of the plate and allowed to incubate overnight. Standard curves were constructed by adding serial dilutions of purified carp VTG (0.1 to 2.0  $\mu$ g/ml) to undiluted male serum diluted in the same range as the fish from the St. Croix River reference area (1:500 to 1:5000) and added



**Figure 2.** (A) SDS-PAGE separation of serum samples from fish collected from the sewage effluent channel (STP) stained with Coomassie Brilliant Blue and (B) Western blot disclosure of vitellogenin in the same samples using monoclonal antibody HL 1147 (2D3-3A9). Lanes containing fish plasma are numbered sequentially 1–17, from left to right, and correspond to samples 1–17 in Table 1. Sex of fish is designated by M (male) or F (female). Lanes labeled MW contain molecular weight markers corresponding to 220 kDa (myosin), 97.4 kDa (phosphorylase b), 66 kDa (bovine serum albumin), 46 kDa (ovalbumin), 30 kDa (carbonic anhydrase), and 21.5 kDa (trypsin inhibitor).

to the wells as indicated above. The next day, the plates were washed in TBST and then incubated with 50  $\mu$ l/well rabbit anti-VTG polyclonal antisera (UF 114) diluted 1:500 to disclose the VTG captured by the monoclonal antibody in the first step; this incubation was done at room temperature for 2 hr. The polyclonal antiserum was in turn disclosed by a 1:1000 dilution of a third antibody, goat anti-rabbit IgG, linked to alkaline phosphatase. As above, this incubation was conducted for 2 hr at room temperature. After a final series of three washes with TBST, 100  $\mu$ l of substrate solution (*p*-nitrophenyl phosphate in carbonate buffer, pH 9.6) was added to each well and incubated for 30 min. The intensity of the yellow color that developed was quantified at 405 nm with an automated ELISA reader. VTG concentrations were calculated based on the standard curve after subtracting values for the male control serum.

### Steroid Analysis

All samples and standards for radioimmunoassay analysis were prepared in duplicate and were analyzed in a single assay. Samples [75  $\mu$ l for estradiol 17 $\beta$ -estradiol ( $E_2$ ) and 25  $\mu$ l for testosterone (T)] were extracted twice with 4 ml diethyl ether. Ether extracts were then dried under a constant air stream. For T determinations, testosterone antiserum (T3-125; Endocrine Sciences, Calabasas Hills, CA) was used at a final concentration of 1:14,400. Cross-reactivities of this antiserum to other ligands are as follows: dihydrotestosterone, 44%;  $\Delta$ -1-testosterone, 41%;  $\Delta$ -1-dihydrotestosterone, 18%; 5  $\alpha$ -androstane-3 $\beta$ , 17 $\beta$ -diol, 3%; 4-androstene-3 $\beta$ , 17 $\beta$ -diol, 2.5%;  $\Delta$ -4-androstenedione, 2%; 5 $\beta$ -androstane-3 $\beta$ , 17 $\beta$ -diol 1.5%; estradiol, 0.5%; all other ligands <0.2%. For  $E_2$  determinations, estradiol antiserum (E26-47; Endocrine Sciences) was used at a final concentration of 1:95,000. Cross-reactivities of this antiserum to other ligands are as follows: estrone, 1.3%; estriol, 0.6%; 16-keto-estriol, 0.2%; all other ligands <0.2%. Tritiated T (TRK.921) and  $E_2$  (TRK.587; Amersham International, Arlington Heights, IL) labels were used at 10,000 cpm per tube. To reduce nonspecific binding, bovine serum albumin (BSA; Sigma Chemical Company, St. Louis, MO) was added at a final concentration of 1.9%. Antisera, label, and BSA were diluted in assay buffer (0.05 M borate buffer, pH 8.0 with 10 N NaOH). Final assay volume was 500  $\mu$ l. T and  $E_2$  concentrations of 0, 1.563, 3.125, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 pg/tube were used to generate the standard curve. Interassay variations were 4.08% for the T assay and 2.99% for the  $E_2$  assay. Samples were incubated at 4°C for 18

hr. Bound-free separation was achieved by adding 500  $\mu$ l of 5% charcoal/0.5% dextran in 0.5 M PBS, followed immediately by a 30-min centrifugation at 1500  $\times$  g. The supernatant (500  $\mu$ l) was diluted with Scintiverse BD (Fisher Scientific, Pittsburgh, PA) and counted on a Beckman scintillation counter (Beckman Instruments, Palo Alto, CA). Hormone concentrations were determined with a commercially available software package (Beckman Immunofit; Beckman Instruments).

### Statistics

To test for significant differences among collection sites, we analyzed the plasma steroid hormone concentrations using one-way ANOVA. If an overall significance was detected, Scheffe's *S*-tests were performed to determine differences between specific pairs. Prior to analysis, the raw data for serum T and  $E_2$  concentrations were log transformed to achieve homogeneity of variance. Steroid hormone data were also examined as an estrogen/androgen (E/A) ratio. These data were arc sine transformed prior to analysis. To determine any relationship between serum steroid and VTG concentrations, a Pearson's product moment correlation analysis was performed. All data are reported as mean  $\pm$  1 standard error (SE). Probability of significance was set at  $p \leq 0.05$ . All statistical analyses were conducted with Stat-View II software (Abacus Concepts, Berkeley, CA).

## Results

### Serum Vitellogenin Concentrations

Gel electrophoresis and Western blot analysis is a very sensitive method to detect the presence of VTG in serum. Mab HL 1147 (2D3-3A9) reacts specifically with carp VTG present in vitellogenic females (or  $E_2$ -induced males), but not with control males. In Figure 2B, the prominent VTG band in Lane 1 corresponds to 150 kDa. In some lanes, VTG is also expressed as a higher MW band of 180 kDa. The bands appearing below 150 kDa correspond to about 1% of the VTG protein and are breakdown products of VTG visualized by the high sensitivity of this assay. When male control serum is probed by Western blot, no bands appear, indicating that these bands are not the result of cross-reactivity with normal serum proteins (data not shown). Plasma samples of all fish collected in this study were analyzed by gel electrophoresis and Western blot analysis. While most of the female fish collected at all sites had measurable amounts of VTG, both female and male fish collected in the sewage effluent canal (STP) had visible lev-

els of VTG by Western blot analysis (Fig. 2B). The corresponding lanes in the Coomassie blue stained gels (Fig. 2A) show that the equivalent amount of serum protein was loaded into each lane. As observed from the blot, 6 of the 7 females had high levels of VTG, while one had a lower level. Seven of the 10 males had visible levels, with 6 of those 7 in the range represented by the females. The lesser bands that are visible on the blot are degradation products of VTG and are most prominent in those samples with high VTG concentrations. The capture-ELISA assay was performed on the samples from the sewage effluent canal (STP) to quantitate the VTG concentrations in those fish (Table 1). One female showed a low level of VTG, while the remaining females ranged from 240 to 1357  $\mu$ g/ml. Two males showed no VTG present, two showed low levels, and the remaining six were within the range of female VTG concentrations.

### Serum Steroid Concentrations

All fish had detectable concentrations of the sex steroid hormones T and  $E_2$ . We observed no significant differences in serum  $E_2$  ( $F = 1.18$ ;  $df = 4,40$ ;  $p = 0.34$ ) concentrations among males from different capture sites. In contrast, serum T concentrations varied significantly among sites ( $F = 2.89$ ;  $df = 4,40$ ;  $p = 0.03$ ), with males from the sewage effluent canal (STP) and the Minnesota River (MNR) having the lowest concentrations (Table 2). Hormone concentrations were not correlated to body size in male fish (T,  $r^2 = 0.056$ ;  $E_2$ ,  $r^2$

**Table 1.** Serum vitellogenin (VTG), 17 $\beta$ -estradiol ( $E_2$ ), and testosterone (T) concentrations in serum of 10 male and 7 female carp (*Cyprinus carpio*)

Sample Number	Sex	VTG ( $\mu$ g/ml)	$E_2$ (pg/ml)	T (pg/ml)
1	F	240	98.7	567.6
2	M	10,000	IS	IS
3	M	165	145.6	2598.4
4	M	30	76.8	1372.0
5	F	1170	116.9	345.1
6	F	35	432.5	620.4
7	M	0	256.0	14916.0
8	F	7500	215.9	1446.0
9	M	150	105.0	1446.4
10	M	10	140.4	1474.8
11	M	5	96.5	1402.8
12	F	1357	381.2	768.4
13	M	340	136.9	463.2
14	M	0	55.0	2024.0
15	F	285	118.5	457.6
16	F	1360	153.5	831.6
17	M	430	116.4	1057.2

Fish were collected from the sewage effluent canal of the St. Paul, Minnesota, metropolitan sewage treatment plant. Values are averages of duplicate determinations. IS, insufficient sample.

= 0.022). As observed for serum T, a significant difference was observed among collection sites when the data were analyzed as E/A ratios ( $F = 4.73$ ;  $df = 4,40$ ;  $p = 0.003$ ). Male fish collected in the effluent stream of the sewage treatment plant and from the Minnesota River exhibited significantly elevated E/A ratios compared to fish obtained from the reference site (St. Croix River). Reduced serum T, rather than increased serum estrogen, was responsible for that significant increase (Table 2). No statistically significant relationship was observed between either the serum  $E_2$  concentrations or the E/A ratios and serum VTG concentrations in male fish. However, the relationship between E/A ratio and serum VTG concentration did border on significant ( $r^2 = 0.434$ ;  $p = 0.054$ ), suggesting that this relationship should be considered in future research.

Although this study focused primarily on males, adequate numbers of females were obtained from two sites, STP and MRP-2. Serum sex steroids in these females did not vary significantly between sites, but serum T concentrations were a quarter of that observed in males, whereas plasma  $E_2$  concentrations were 2–4 times greater than those detected in males (Table 2). The E/A ratio ( $\times 100$ ) for females from STP was  $32.8 (\pm 7.6)$  while females from MRP-2 averaged  $60.9 (\pm 6.6)$ . The disparity in female E/A ratio was due to variation in serum  $E_2$  concentrations, the opposite of that observed in the males. The E/A ratios for females were greatly elevated compared to those observed in males from any site.

## Discussion

Our results show a pronounced estrogenic effect (VTG production in male carp) associated with exposure to municipal sewage effluent (Fig. 2). Serum VTG concentration was elevated without a coincident significant increase in the circulating concentration of the endogenous estrogen,  $E_2$  (Table 2). However, circulating T was reduced and the E/A ratio was elevated in male fish caught in the effluent channel.

Vitellogenin induction was not observed at any other sampling location, even in fish collected between 3 and 17 miles downstream of the effluent channel (MRP-2). No attempt was made to establish a gradient of VTG induction away from the effluent channel due to the diluting effect associated with an abnormally high flow rate of the Mississippi River in 1995 (160–300% of normal in May through October). The presence of VTG in the serum of male carp suggests that there is an estrogenic component of the sewage discharge. Similar observations have been reported with caged fish in Great Britain rivers receiving domestic sewage (14,30). Purdom et al. (14) suggested that the most likely estrogenic substances in domestic sewage are ethynyl estradiol ( $EE_2$ ) and alkylphenol polyethoxylates (APEs); however, a wide range of environmental contaminants possessing estrogenic activity have been reported and may be associated with domestic sewage (31,32). Estrogens have been measured by radioimmunoassay in the effluent of a modern sewage treatment facility and shown to vary from 7 to 52 ng/l (33). Male sheephead minnows can produce measurable quantities of VTG after a 3-day exposure to  $E_2$  at 10 ng/l (Folmar, unpublished observations). Purdom et al. (14) also showed that  $EE_2$  at 1–10 ng/l could induce a vitellogenic response in caged rainbow trout. Alkylphenol polyethoxylates are produced worldwide at >300,000 tons annually, and as much as 60% of that ends up in the aquatic environment (34). These chemicals have been shown to bind to the estrogen receptor of fish and mammals (7,35) and are capable of inducing VTG production in fish hepatocyte cell culture (7,13). Additionally, a wide array of chemicals, including pesticides and plasticizers commonly found in waste water, stimulate VTG production from fish hepatocytes *in vitro* (12). In general, there appears to be little similarity in structure among many of the documented estrogenic chemicals (36), but a *para*-substituted phenolic group (37) and structural rigidity (38,39) appear to

enhance estrogenicity. Serum T concentrations were lowest in male fish collected from the effluent channel and the Minnesota River. No males from any location showed increased serum  $E_2$  concentrations, nor were those concentrations elevated when compared to females. Males from the effluent channel had detectable serum VTG concentrations, but male fish from the Minnesota River did not; this suggests that the elevated E/A ratios were not responsible for that increase. These findings indicate that vitellogenesis in the male fish from the sewage effluent channel was not induced by endogenous estrogens. Although we did not determine which chemical(s) was responsible for depressing serum T concentrations, phytoestrogens such as  $\beta$ -sitosterol found in pulp mill effluent (10,40) are capable of dramatically reducing serum T concentrations by altering cholesterol availability (41). The E/A ratio appears to be a sensitive marker of abnormal serum sex steroid concentrations; however, when taken alone, the E/A ratio has little functional significance because normal ranges for this parameter have not been established for most wildlife species. Circulating sex steroid concentrations vary significantly in fish and other vertebrates throughout the annual reproductive cycle (42). Although the E/A ratio or serum sex steroid concentrations can be informative of contaminant exposure during embryonic development (43,44) and adulthood (10,41,42), samples must be collected in such a way as to minimize naturally occurring variation. Used in collaboration with serum VTG analysis, the serum sex steroid concentrations have provided important insight into the mechanism by which male fish are induced to express VTG. Although we have focused on induced VTG production in male fish exposed to chemical contaminants, earlier field studies reported either no change [winter flounder (45)] or decreases [English sole (46,47)] in serum VTG levels in female fish exposed to polynuclear aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). Similarly, several laboratory studies have shown decreases or no change in  $E_2$  and VTG synthesis in female fish exposed to acid, metals, cyanide, pesticides, PCBs, and PAHs (48–54). In females, elevated levels of various estrogen mimics could stimulate negative feedback in gonadotropin secretion, resulting in suppressed synthesis of endogenous estrogens, and thus, reduced VTG synthesis. Likewise, estrogen mimics that are weaker could occupy hepatic estrogen receptors but stimulate a lower level of VTG gene expression—an antiestrogenic effect. Our results show that the sewage

**Table 2.** Serum concentrations (mean  $\pm$  1 SE) of testosterone (T) and 17 $\beta$ -estradiol ( $E_2$ ) in adult post-spawning male and female carp (*Cyprinus carpio*) collected from five locations

Site	Number (M/F)	T (ng/ml)		$E_2$ (pg/ml)	
		Male	Female	Male	Female
STP	10/7	$2.97 \pm 1.5^a$	$0.72 \pm 0.1$	$125.4 \pm 19.2$	$216.7 \pm 51.5$
MRP-2	10/10	$4.16 \pm 0.8^b$	$1.25 \pm 0.1$	$125.9 \pm 7.3$	$750.5 \pm 89.6$
MRP-3	11/0	$5.13 \pm 0.7^b$	ND	$147.3 \pm 14.3$	ND
MNR	10/0	$2.92 \pm 0.4^a$	ND	$133.6 \pm 13.7$	ND
SCR	5/0	$6.41 \pm 2.0^b$	ND	$105.4 \pm 22.0$	ND

ND, no data available; STP, channel below the St. Paul metropolitan sewage treatment plant; MRP-2, Mississippi River Pool #2; MRP-3, Mississippi River Pool #3; MNR, Minnesota River; SCR, St. Croix River.

<sup>a,b</sup>Values with different superscripts are significantly different at  $p \leq 0.05$ .

effluent did not enhance endogenous estrogen synthesis, but it appears to act directly on the estrogen receptor (ER) or possibly through the disruption of other hormones such as the thyroid hormones, growth hormone, prolactin, or cortisol known to enhance hepatic yolk protein synthesis (55–58). For example, a variety of pesticides, PCBs, and acidified water have been shown to alter circulating concentrations of triiodothyronine ( $T_3$ ) and thyroxine ( $T_4$ ) in fish (59–62). Thyroid hormones are known to potentiate estrogen induction of VTG mRNA and stimulate synthesis of estrogen receptors in frog hepatocytes (58). Likewise, contaminants at higher concentrations are capable of producing a generalized stress response with accompanying increases in circulating levels of cortisol. Cortisol has been shown to cause rapid but transient increases in male *Oreochromis* VTG mRNA (63), decreased  $E_2$  secretion in isolated rainbow trout ovarian follicles (64), increased serum binding capacity, decreased cytosolic and nuclear binding sites with no change in circulating  $E_2$  levels in immature female rainbow trout (65), and suppressed plasma VTG concentrations in female catfish (66). Future studies must begin to examine a wide range of hormones to determine the various mechanisms by which males are stimulated to express elevated serum concentrations of VTG. This study confirms similar observations from studies with caged fish in Great Britain (23,24) and indicates that similar problems exist in the United States, even with state-of-the-art sewage treatment plants. Future studies should focus on how such changes in serum sex steroid and VTG concentrations relate to reproductive dysfunction in individuals and consequential changes in populations.

## REFERENCES

- Bitman J, Cecil HC. Estrogenic activity of DDT analogs and polychlorinated biphenyls. *J Agric Food Chem* 18:1108–1112 (1970).
- Nelson JA, Struck RF, James R. Estrogenic activities of chlorinated hydrocarbons. *J Toxicol Environ Health* 4:325–339 (1978).
- Colborn T, Clement C (eds) Chemically-induced alterations in sexual and functional development: the wildlife/human connection. Princeton, NJ:Princeton Scientific Publishing, 1992.
- Rolland R, Gilbertson M, Colborn T (eds). Environmentally induced alterations in development: a focus on wildlife. *Environ Health Perspect* 103(suppl 4), 1995.
- Estrogens in the environment III: global health implications. *Environ Health Perspect* 103(suppl 7), 1995.
- Wester PW. Histopathological effects of environmental pollutants  $\beta$ -HCH and methyl mercury on reproductive organs in freshwater fish. *Comp Biochem Physiol* 100C:237–239 (1991).
- White R, Jobling S, Hoare SA, Sumpter JP, Parker MG. Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrinology* 135:175–182 (1994).
- Pelissier C, Bennetau B, Babin P, Le Menn F, Dunogues J. The estrogenic activity of certain phytoestrogens in the Siberian sturgeon *Acipenser baeri*. *J Steroid Biochem Mol Biol* 38:293–299 (1991).
- Pelissier C, Flouriot G, Foucher JL, Bennetau B, Dunogues J, Le Gac F, Sumpter JP. Vitellogenin synthesis in cultured hepatocytes: an *in vitro* test for the estrogenic potency of chemicals. *J Steroid Biochem Mol Biol* 44:263–272 (1993).
- Munkittrick KR, Port CB, Van Der Kraak GJ, Smith IR, Rokosh DA. Impact of bleached kraft mill effluent on population characteristics, liver MFO activity, and serum steroids of the Lake Superior white sucker (*Catostomus commersoni*) population. *Can J Fish Aquat Sci* 48:1–10 (1991).
- Van Der Kraak GJ, Munkittrick KR, McMaster ME, Port CB, Chang JP. Exposure to bleached kraft pulp mill effluent disrupts the pituitary–gonadal axis of white sucker at multiple sites. *Toxicol Appl Pharmacol* 115:224–233 (1992).
- Jobling S, Reynolds T, White R, Parker MG, Sumpter JP. A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. *Environ Health Perspect* 103:582–587 (1995).
- Jobling S, Sumpter JP. Detergent components in sewage effluent are weakly estrogenic to fish: an *in vitro* study using rainbow trout hepatocytes. *Aquat Toxicol* 27:361–372 (1993).
- Purdum CE, Hardiman PA, Bye VJ, Eno NC, Tyler CR, Sumpter JP. Estrogenic effects of effluents from sewage treatment works. *Chem Ecol* 8:275–285 (1994).
- Wallace R. Vitellogenesis and oocyte growth in non-mammalian vertebrates. In: *Developmental biology*, vol 1 (Browder LW, ed). New York:Plenum Press, 1985;127–177.
- Specker J, Sullivan CV. Vitellogenesis in fishes: status and perspectives. In: *Perspectives in comparative endocrinology* (Davey KG, Peter RG, Tobe SS, eds). Ottawa:National Research Council of Canada, 1994;304–315.
- Copeland PA, Sumpter JP, Walker TK, Croft M. Vitellogenin levels in male and female rainbow trout (*Salmo gairdneri* Richardson) at various stages of the reproductive cycle. *Comp Biochem Physiol* 83B:487–493 (1986).
- Copeland PA, Thomas P. The measurement of plasma vitellogenin levels in a marine teleost, the spotted seatrout (*Cynoscion nebulosus*), by homologous radioimmunoassay. *Comp Biochem Physiol* 91B:17–23 (1988).
- Ding JL, Hee PL, Lam TJ. Two forms of vitellogenin in the plasma and gonads of male *Oreochromis aureus*. *Comp Biochem Physiol* 93B:363–370 (1989).
- Goodwin AE, Grizzle JM, Bradley JT, Estridge BH. Monoclonal antibody-based immunoassay of vitellogenin in the blood of male catfish (*Ictalurus punctatus*). *Comp Biochem Physiol* 101B:441–446 (1992).
- Kishida M, Specker J. Vitellogenin in tilapia (*Oreochromis mossambicus*): induction of two forms by estradiol, quantification in plasma and characterization in oocyte extract. *Fish Physiol Biochem* 12:171–182 (1993).
- Maitre JL, LeGuellec C, Derrien S, Tenniswood M, Valotaire Y. Measurement of vitellogenin from rainbow trout by rocket immunoelectrophoresis: application to the kinetic analysis of estrogen stimulation in the male. *Can J Biochem Cell Biol* 63:982–987 (1985).
- Ho S-M. Endocrinology of vitellogenesis. In: *Hormones and reproduction in fishes, amphibians and reptiles*. (Norris DO, Jones RT, eds). New York:Plenum Press, 1987;413–421.
- LeGuellec K, Lawless K, Valotaire Y, Kress M, Tenniswood M. Vitellogenin gene expression in male rainbow trout (*Salmo gairdneri*). *Gen Comp Endocrinol* 71:359–371 (1988).
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685 (1970).
- Schagger H, von Jagow G. Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 166:368–379 (1987).
- Denslow ND, Patten B, Nguyen HP. Problott: a membrane for electroblotting peptides after enzymatic digestion in gel slices. In: *Protein analysis renaissance*. Foster City, CA:Applied Biosystems, 1993;33–36.
- Johnson DA, Gautsch JW, Sportsman JR, Elder JH. Improved technique utilizing non-fat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. *Gene Anal Tech* 1:3–8 (1984).
- Peterson GL. Determination of total protein. *Methods Enzymol* 91:95–121 (1993).
- Sumpter JP, Jobling S. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environ Health Perspect* 103(suppl 7):173–178 (1995).
- Guillette LJ Jr, Arnold SF, McLachlan JA. Ecoestrogens and embryos—is there a scientific basis for concern? *Anim Reprod Sci* 42:13–24 (1996).
- Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Olea Serrano F. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ Health Perspect* 103(suppl 7):113–122 (1995).
- Shore LS, Gurevitz M, Shemesh M. Estrogen as an environmental pollutant. *Bull Environ Contam Toxicol* 51:361–366 (1993).
- Naylor GC, Mierure JP, Weeks JA, Castaldi FJ, Romano RR. Alkylphenol ethoxylates in the environment. *J Am Oil Chem Soc* 69:695–703 (1992).
- Arnold SF, Robinson MK, Notides AC, Guillette LJ Jr, McLachlan JA. A yeast estrogen screen for examining the relative exposure of cells to natural and xenoestrogens. *Environ Health Perspect* 104:544–548.
- McLachlan JA. Functional toxicology: a new approach to detect biologically active xenobiotics. *Environ Health Perspect* 101:386–387 (1993).
- Jordan VC, Mittal S, Gosden B, Koch R, Lieberman ME. Structure–activity relationships of estrogens. *Environ Health Perspect* 61:97–110 (1985).
- Korach KS, Sarver P, Chae K, McLachlan JA, McKinney JD. Estrogen receptor-binding activity of polychlorinated hydroxybiphenyls: conformationally restricted structural probes. *Mol Pharmacol* 33:120–126 (1988).

39. McKinney JD, Waller CL. Polychlorinated biphenyls as hormonally active structural analogues. *Environ Health Perspect* 102:290–297 (1994).
40. McMaster ME. Mechanisms of reproductive dysfunction in white sucker (*Catostomus commersoni*), brown bullhead (*Ictalurus nebulosus*), and goldfish (*Carassius auratus*) exposed to various pulp mill effluents and polycyclic aromatic hydrocarbon contaminants [Ph.D. Thesis]. University of Guelph, Guelph, Ontario, Canada, 1995.
41. MacLachy DL, Van Der Kraak GJ. The phytoestrogen  $\beta$ -sitosterol alters the reproductive endocrine status of goldfish. *Toxicol Appl Pharmacol* 134:305–312 (1995).
42. van Tienhoven A. Reproductive physiology of vertebrates. Ithaca, NY: Cornell University Press, 1982.
43. Guillette LJ Jr, Gross TS, Gross DA, Rooney AA, Percival HF. Gonadal steroidogenesis *in vitro* from juvenile alligators obtained from contaminated and control lakes. *Environ Health Perspect* 103 (suppl 4):31–36 (1995).
44. Guillette LJ Jr, Gross TS, Masson GR, Matter JM, Percival HF, Woodward AR. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environ Health Perspect* 102: 680–688 (1994).
45. Pereira JJ, Mercado-Allen R, Kuropat C, Luedke D, Sennefelder G. Effect of cadmium accumulation on serum vitellogenin levels and hepatosomatic and gonadosomatic indices of winter flounder (*Pleuronectes americanus*). *Arch Environ Contam Toxicol* 24:427–431 (1993).
46. Casillas E, Misitano D, Johnson LL, Rhodes LD, Collier TK, Stein JE, McCain BB, Varanasi U. Inducibility of spawning and reproductive success of female English sole (*Parophrys vetulus*) from urban and nonurban areas of Puget Sound, Washington. *Mar Environ Res* 31:99–122 (1991).
47. Johnson LL, Casillas E, Sol S, Collier TK, Stein JE, Varanasi U. Contaminant effects on reproductive success in selected benthic fish. *Mar Environ Res* 35:165–170 (1993).
48. Chen TT, Reid PC, Van Beneden R, Sonstegard RR. Effect of Aroclor 1254 and mirex on estradiol-induced vitellogenin production in juvenile rainbow trout, *Salmo gairdneri*. *Can J Fish Aquat Sci* 43:169–173 (1986).
49. Tam WH, Fryer JN, Valentine B. Reduction in oocyte production and gonadotrope activity, and plasma levels of estrogens and vitellogenin, in brook trout exposed to low environmental pH. *Can J Zool* 68:2468–2476 (1990).
50. Povlsen AF, Korsgaard B, Bjerregaard P. The effect of cadmium on vitellogenin metabolism in estradiol-induced flounder (*Platichthys flesus* (L.)) males and females. *Aquat Toxicol* 17:253–262 (1990).
51. Mount DR, Hockett JR, Gern WA. Effect of long-term exposure to acid, aluminum and low calcium on adult brook trout *Salvelinus fontinalis*. 2. Vitellogenesis and osmoregulation. *Can J Fish Aquat Sci* 45:1633–1642 (1988).
52. Chakravorty S, Lal B, Singh TP. Effect of endosulfan (thiodan) on vitellogenesis and its modulation by different hormones in the vitellogenic catfish *Clarias batrachus*. *Toxicology* 75:191–198 (1992).
53. Ruby SM, Idler DR, So YP. Plasma vitellogenin,  $17\beta$ -estradiol,  $T_3$  and  $T_4$  levels in sexually maturing rainbow trout *Oncorhynchus mykiss* following sublethal HCN exposure. *Aquat Toxicol* 26:91–102 (1993).
54. Monosson E, Fleming WJ, Sullivan CV. Effects of the planar PCB 3,3',4,4'-tetrachlorobiphenyl (TCB) on ovarian development, plasma levels of sex steroid hormones and vitellogenin, and progeny survival in the white perch (*Morone americana*). *Aquat Toxicol* 29:1–19 (1994).
55. Carnevali O, Mosconi G, Yamamoto K, Kobayashi T, Kikuyama S, Polzonetti-Magni AM. Hormonal control of *in vitro* vitellogenin synthesis in *Rana esculenta* liver: effects of mammalian and amphibian growth hormone. *Gen Comp Endocrinol* 88:406–414 (1992).
56. Carnevali O, Mosconi G, Yamamoto K, Kobayashi T, Kikuyama S, Polzonetti-Magni AM. *In vitro* effects of mammalian and amphibian prolactins on hepatic vitellogenin synthesis in *Rana esculenta*. *J Endocrinol* 137:383–389 (1993).
57. Carragher JF, Sumpter JP, Pottinger TG, Pickering AD. The deleterious effects of cortisol implantation on reproductive function in two species of trout, *Salmo trutta* L. and *Salmo gairdneri* Richardson. *Gen Comp Endocrinol* 76:310–321 (1989).
58. Rabelo EM, Tata JR. Thyroid hormone potentiates estrogen activation of vitellogenin genes and autoinduction of estrogen receptor in adult *Xenopus* hepatocytes. *Mol Cell Endocrinol* 96:37–44 (1993).
59. Sinha N, Lal B, Singh TP. Pesticide induced changes in circulating thyroid hormones in the freshwater catfish *Clarias batrachus*. *Comp Biochem Physiol* 100C:107–110 (1991).
60. Folmar LC, Dickhoff WW, Zaugg WS, Hodgins HO. The effects of Aroclor 1254 and No 2 fuel oil on smoltification and seawater adaptation of coho salmon (*Oncorhynchus kisutch*). *Aquat Toxicol* 2:291–299 (1982).
61. Leatherland JF, Sonstegard RA. Lowering of serum thyroxine and triiodothyronine levels in yearling coho salmon, *Oncorhynchus kisutch*, by dietary mirex and PCBs. *J Fish Res Board Can* 34:1285–1289 (1978).
62. Brown SB, Evans RE, Hara TJ. Interrenal, thyroidal, carbohydrate and electrolyte responses in rainbow trout (*Salmo gairdneri*) during recovery from the effects of acidification. *Can J Fish Aquat Sci* 43:714–718 (1986).
63. Ding JL, Lim EH, Lam TJ. Cortisol-induced hepatic vitellogenin mRNA in *Oreochromis aureus* (Steindachner). *Gen Comp Endocrinol* 96:276–287 (1994).
64. Carragher JF, Sumpter JP. The effect of cortisol on the secretion of sex steroids from cultured ovarian follicles of rainbow trout. *Gen Comp Endocrinol* 77:403–407 (1990).
65. Pottinger TG, Pickering AD. The effect of cortisol administration on hepatic and plasma estradiol-binding capacity in immature female rainbow trout (*Oncorhynchus mykiss*). *Gen Comp Endocrinol* 80:264–273 (1990).
66. Sundararaj BI, Nath P. Steroid-induced synthesis of vitellogenin in the catfish *Heteropneustes fossilis* (Bloch). *Gen Comp Endocrinol* 43:201–210 (1981).

## Postdoctoral positions available

- Molecular Neurobiology
- Characterization of Receptor–Ligand Interactions
- Xenobiotic Transport Mechanisms
- Renal Transport Physiology
- Cell Adhesion in Metastasis
- Xenobiotic Metabolism
- Mechanistic Aspects of DNA Mismatch Repair on Recombination

- Molecular and Cellular Biology
- Mechanisms by which Organisms Produce Mutations
- Molecular Mechanisms of Respiratory Diseases
- Molecular Biology and Fatty Acid Biochemistry

See page 1120 for more information.